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A slow release formulation of insulin as a treatment for osteoarthritis

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Summary

Objective: To examine the potential of insulin, in a sustained delivery system, as a treatment for arthritis.

Design: The effect of insulin on matrix synthesis, matrix breakdown, and nitric oxide production in primary cartilage explants was examined. The activity of insulin on diseased cartilage from Dunkin Hartley guinea pigs, diabetic mice, and osteoarthritic patients was measured. The specificity of insulin stimulation was compared to that of IGF-I using osteoblasts and fibroblasts. Finally, the stability of insulin in a biologically relevant system was tested, and a slow-release formulation of insulin was developed and characterized.

Results: In articular cartilage explants, insulin stimulated proteoglycan (PG) synthesis, inhibited PG release and nitric oxide production, and overcame the detrimental effects of interleukin 1 (IL-1). The mechanism whereby insulin decreased matrix breakdown was through inhibition of aggrecanase activity. Insulin was active on cartilage at concentrations at which insulin does not cross-react with insulin-like growth factor I (IGF-I) receptors nor stimulate proliferation of other cells types. The response of cartilage to insulin did not diminish with age or disease. Insulin stimulated matrix synthesis in osteoarthritic cartilage and local treatment with insulin overcame endogenous suppression of matrix synthesis in diabetic cartilage. Poly-lactic-coglycolic acid (PLGA) was found to be an effective carrier for delivery of insulin, and PLGA-Insulin was active on articular cartilage *in vitro* and *in vivo*.

Conclusions: As the incidence of arthritis increases with the aging population, an effective therapy to induce repair of cartilage is needed. Based on its biological activities, insulin appears to be an attractive protein therapeutic candidate. Maximum insulin effectiveness may require a sustained delivery system.

Key words: Osteoarthritis, Cartilage, Insulin, Growth factors, Matrix breakdown.

Introduction

Osteoarthritis (OA) is characterized by loss of cartilage matrix molecules i.e. proteoglycans (PG) and collagens. Because of the limited replicative capacity of mature chondrocytes and the avascular nature of cartilage, mature cartilage has little ability to effectively repair itself[1]. Growth factors modulate chondrocyte proliferation, differentiation, migration, and matrix turnover[2]. Treatment with growth factors alone, or as part of a device, could be used to promote *in vivo* repair cartilage or to promote expansion of cells *ex vivo*. Several secreted peptides, including members of the insulin-like growth factor (IGF), bone morphogenetic protein (BMP), and transforming growth factor beta (TGF-β) families, have the potential to induce cartilage repair[1,2].

IGF-I is of particular interest, since insufficiency of IGF-I may have an etiologic role in the development of osteoarthritis[2,3]. More specifically, serum IGF-I concentrations can be lower in osteoarthritic patients relative to controls[3]. In addition, chondrocyte responsiveness to IGF-I decreases with age and disease, a phenomenon which may be due to increased production of IGF binding proteins (IGF-BPs)[3–8]. Thus, decreased availability of, and responsiveness to, IGF-1 may contribute to loss of homeostasis and cartilage degeneration with increasing age and disease[3–8].

Insulin can stimulate the growth of a number of cell types[9,10], including chondrocytes from hypophysectomized rats[11,12]. Most of the earliest studies with insulin were performed in whole animals, organs, or tissues[11–14]. In chondrocytes derived from chick embryo sternal cartilage, insulin stimulates mucopolysaccharide synthesis, amino acid uptake, and promotes a positive nitrogen balance[15]. Similarly, insulin increases proteoglycan synthesis in rat tumor cells derived from a Swarm rat chondrosarcoma[16]. Levels of insulin as high as 10 μg/ml were used in many of these studies. At such high concentrations, insulin binds to, and activates, the IGF-1 receptor, thus mimicking the effects of IGF-I itself[17]. The observed physiological effects could therefore be the result of IGF-I receptor signaling.

We have tested the effect of insulin on articular cartilage at concentrations at which activation of IGF-I receptors does not occur[17]. At these low concentrations (≤10 nM), insulin decreased matrix breakdown by inhibiting aggrecanase activity. Insulin also increased matrix synthesis, inhibited nitric oxide production, and blocked the detrimental effects of IL-1 on articular cartilage. Since insulin was not stable in articular cartilage cultures, we created and characterized a slow-release formulation of insulin. We show that our slow-release formulation was efficacious *in vitro* and *in vivo*. Finally, our data suggest that a decrease in insulin is associated with defective matrix

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synthesis, and that insulin may have advantages over other therapeutic growth factors, such as IGF-I.

Material and methods

REAGENTS

Recombinant human interleukin 1alpha, IL-1α, purchased from R&D systems (Minneapolis, MN), was resuspended in phoshate buffered saline (PBS) 0.1% and bovine serum albumin (BSA) prior to use. Recombinant human IGF-I and recombinant human des-(1-3)-IGF-I were made at Genentech. Recombinant human insulin was from Intergen (Purchase, New York).

ANIMALS

Mice and male Dunkin Hartley guinea pigs were obtained from Charles River Laboratories (Wilmington, MA). All animals were maintained and treated according to guidelines for care and use of animals by the Institutional Animal Care and Use Committee (IACUC).

CHONDROCYTE PREPARATION

Articular cartilage was aseptically dissected from the metacarpo-phalangeal joints of 4–6-month-old female pigs (University of California Davis, Davis, CA). Cartilage fragments were digested in 0.05% trypsin in serum-free Ham's F12 (F12) for 25 min at 37°C. Cartilage was further digested in 0.3% collagenase B (Boehringer Mannheim. Mannheim, Germany) in F12 media for 30 min at 37°C. Cartilage was then digested overnight in 0.06% collagenase B in F12+10% fetal bovine serum (FBS). Cells were filtered (70 μm nylon filter) and seeded at 8×10^6 in 150 cm^2 flasks in F12 medium+10% FBS.

CULTURING OF CHONDROCYTES

Chondrocytes (prepared as described above) were grown in 150 cm^2 flasks and then were plated on microtiter plates (Falcon microtest 96, flat bottom) at a density of 80,000 cells per well in F12 with antibiotics (10 μg/ml gentamicin, 250 ng/ml amphotericin B, 100 U/ml penicillin/100 μg/ml streptomycin) and 10% FBS. 24 h after plating cells were treated with various factors in serum-free media and cultured for 5 days.

MTT ASSAY

Dimethylthiazol-diphenyltetrazolium bromide, MTT[18], cell proliferation kit was used as per manufacturer's protocol (Boehringer Mannheim).

MEASUREMENT OF PROTEOGLYCANS

To measure proteoglycans, we used a dimethylmethylene blue (DMMB) assay[19], a well-accepted method to measure the amount of proteoglycans in cartilage cultures[20]. As a standard, chondroitin sulfate was used (0.0 to 5.0 μg) from shark cartilage (Sigma, St Louis, MO).

ARTICULAR CARTILAGE EXPLANTS

Articular cartilage was aseptically dissected from the metacarpo-phalangeal joints of 4–6-month-old female pigs, 18–24-month-old cows (Harris Ranch, Selma, CA), or the femorotibial joint of Dunkin Hartley guinea pigs (1–2, 6, or 11 months old). Cartilage was pooled, minced, washed and cultured in bulk for at least 24 h in Explant Media, i.e. serum free low glucose 50:50 DMEM:F12 media with 0.1% BSA, 100 U/ml penicilin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1× GHT, 0.1 mM MEM sodium pyruvate, 20 μg/ml gentamicin (Invitrogen, Carlsbad, CA), 1.25 mg/l amphotericin B, 10 μg/ml transferrin and 5 μg/ml vitamin E. Approximately 50 mg (for porcine and bovine cartilage) or 30 mg (for guinea pig cartilage) of tissue was aliquoted into micronics tubes (Costar, Acton, MA) and incubated for at least 24 h in above media before changing to media without transferrin and vitamin E. Test proteins were then added. Media was harvested and changed at various time points (0, 24, 48, 72 h). For human articular cartilage, tissue was dissected, minced and weighed the same day and test proteins were added the following day. Human cartilage samples from OA patients (54–78 years old) undergoing knee joint replacement because of advanced disease were obtained from CHTN (Cooperative Human Tissue Network, Cleveland, Ohio).

MEASUREMENT OF PROTEOGLYCAN SYNTHESIS IN ARTICULAR CARTILAGE EXPLANTS

At 48 h, ^{35}S-sodium sulfate (ICN Radiochemicals, Irvine, CA) was added to cartilage explants (10 μCi/ml). After an overnight incubation at 37°C, media were used to measure nitric oxide or proteoglycan content. Cartilage pieces were washed two times using explant media. 900 μL (750 μL for human articular cartilage) digestion buffer containing 10 mM EDTA, 0.1 M sodium phosphate and 1 mg/ml proteinase K (Invitrogen) was added to each tube and incubated overnight in a 50°C water bath. Samples of digest were assayed for proteoglycan content. 600 μL of digest was mixed with 600 μL of 10% w/v cetylpyridinium chloride, CPC (Sigma). Samples were spun at 1000 ***g*** for 15 min. Supernatant was removed, and 500 μL formic acid (Sigma) was added to samples to dissolve precipitates. Solubilized pellets were transferred to scintillation vials containing 10 ml scintillation fluid (ICN), and samples were counted. In tests for IGF-I sensitivity, human articular cartilage explant cultures were labeled when test proteins were added. Explant cultures were washed and digested the following day.

WESTERN BLOT ANALYSIS

CPC was added to culture media from bovine explants treated for 3 days to a final concentration of 1% (w/v). Precipitated proteoglycans and proteoglycan fragments were collected by centrifugation. Pellets were washed with 1% (w/v) CPC then dissolved in isopropanol/water (3:2, v/v). Two volumes of ethanol saturated with potassium acetate were added at 4°C, and proteoglycan samples (now as their potassium salts) were collected by centrifugation. Pellets were then washed twice with ethanol, then with ether, and air dried. Proteoglycan samples were dissolved at 10 mg/ml in 0.1 M Tris/acetate, pH 7.0, containing 10 mM EDTA, 10 mM iodoacetamide, 5 mM

phenylmethanesulphonyl fluoride, 0.36 mM pepstatin A, 0.24 unit/ml keratanase I (endo β-galactosidase, Sigma), 0.1 unit/40 mg Keratanase II and 0.12 unit/ml chondroitinase ABC and incubated at 37°C overnight. Digestion was terminated by addition of SDS/PAGE sample buffer and boiling for 3 minutes. Samples were run on 4–12% Tris-glycine SDS/PAGE gradient gels and electroblotted to nitrocellulose membranes (Novex, San Diego, CA), which were probed with 1:1000 dilution of antibodies recognizing either the aggrecanase generated N-terminal neopeptide ARGSV (antibody #71) or the MMP-generated N-terminal IGD neoepitope FFGVG (antibody #247)[21]. Subsequently, membranes were incubated with sheep antirabbit Ig horseradish peroxidase conjugate (Amersham Biosciences, Piscataway, NJ) and aggrecan catabolites were visualized by incubation with the SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 5 min and then exposed.

NITRIC OXIDE ASSAY

Using a fluorometric assay based on the Griess reaction[22], we measured nitrate, a stable end-product of nitric oxide metabolism which is an appropriate indicator of NO synthesis *in vitro*[23], in the media of cultured explants or chondrocytes.

EFFECT OF INSULIN ON CARTILAGE FROM DIABETIC MICE

Eight-week-old female CD-1 mice were injected with 30 or 50 mg/kg streptozotocin (STZ) for 5 consecutive days. Blood glucose levels were measured using a glucose meter (One-Touch, Lifescan, Buckinghamshire, UK). Two months later, patellae were harvested and incubated in Explant Media (see above), in the absence or presence of insulin (100 nM), for 2 days, and labeled with ^{35}S-sulfate (final concentration 30 μCi/ml) overnight. A patella assay was then performed.

PATELLA ASSAY

Patellae assays were performed as previously described[24,25]. Briefly, following labeling with ^{35}S-sulfate (30 μCi/ml), samples were washed three times with phosphate buffered saline (PBS). Samples were then fixed overnight in 10% formalin followed by decalcification of the underlying bone in 5% formic acid. Cartilage was dissected away from the underlying bone, placed in 500 μl of a tissue and gel solubilizer (Solvable) and incubated at 60°C for 1.5 h. Scintillation fluid designed for concentrated alkaline and salt solutions (HIONIC-fluor, Perkin Elmer, Boston, MA) was added (10 ml) to each tube and mixed thoroughly. ^{35}S uptake was then measured using a scintillation counter.

EFFECT OF INSULIN OR IGF-I ON FIBROBLASTS AND OSTEOBLASTS

Mouse fibroblast cell line NIH3T3 and human osteoblast cell line MG-63 were purchased from ATCC (Manassas, VA). In 96-well plates, NIH3T3 cells were seeded at a concentration of 5000/well, and MG63 cells were seeded at a concentration of 1000/well in high glucose DMEM (HGD-MEM) containing 10% FBS, 2 mM L-glutamine, and 100 U/ml penicillin, 100 μg/ml streptomycin. 24 (NIH3T3) or 72 (MG63) hours after seeding, cells were changed into serum free media and test proteins were added. At day 3 (MG63) or day 6 (NIH3T3), cells were analysed by an MTT assay.

STABILITY OF INSULIN

Insulin (10 nM) was incubated at 37°C and 5% CO_2 in explant media alone or with articular cartilage explants. Media was harvested at various timepoints and tested for activity in the insulin kinase receptor activation assay.

INSULIN KINASE RECEPTOR ACTIVATION (KIRA) ASSAY[17]

For cell stimulation, Chinese hamster ovary (CHO) cells transfected with the human insulin receptor were cultured overnight in 96-well plates (Falcon 1270). Cells were then treated with sample in PS/20 medium with 0.5% BSA for 15 min at 37°C. Cells were lysed by the addition of Lysis Buffer (150 mM NaCl, 50 mM HEPES, 0.5% Triton-X 100, 1 mM AEBSF, 10 000 IU/ml Aprotinin, 0.05 mM Leupeptin, and 2 mM sodium orthovanidate). Levels of insulin receptor autophosphorylation in cell lysates were then assessed by enzyme-linked immuno-sorbent assay, ELISA. For ELISA analysis, 96-well plates (Nunc Maxisorp) were coated with capture antibody (antiinsulin receptor, clone 83-7, LabVision, Inc., Fremont, CA) at 2 μg/ml in PBS and then blocked with 0.5% BSA in PBS. Plates were washed three times with wash buffer (PBS with 0.05% Tween-20). Cell lysates (from above) were diluted 1:5 in lysis buffer and incubated on the prepared ELISA plates for 2 h. After washing six times, plates were incubated for 2 h with biotinylated detection antibody (anti-phosphotyrosine, clone 4G10, Upstate Biotechnology, Inc., Waltham, MA) at 0.1 μg/ml in Assay Buffer (PBS with 0.5% BSA, 0.05% Tween-20 and 5 mM EDTA). Plates were washed six times and incubated for 1 h with Streptavidin/HRP (Amdex, Denmark) in Assay Buffer. Plates were washed six times and incubated with tetramethyl benzidine substrate solution (Kirkegaard and Perry). After sufficient color development, the reaction was quenched with 1.0 N H_3PO_4, and the absorbance at 450 nm was read.

SLOW-RELEASE FORMULATIONS OF INSULIN

Microsphere formulations: the Zn:human insulin (HI) hexamer ratio was altered—formulation I–(2:1) and formulation II–(4:1)—while the protein loading remained constant (approximately 5.0%) in the microsphere formulations prepared. The molar ratio of lactide to glycolide in all polymers was kept constant at 50:50. All microsphere formulations were prepared using D,L- poly lactic-coglycolic acid (PLGA) obtained from Boehringer Ingelheim (Ingelheim, Germany; RG502H; 0.2 dl/g, 8 kD). Recombinant human insulin (Eli Lilly, Indianapolis, IN) was encapsulated into PLGA microspheres using a cryogenic, non-aqueous process[26].

The above human insulin formulations were first spray-freeze-dried in preparation for insertion into the microspheres. To do this, the above formulations were atomized through an ultrasonic nozzle (Sono-Tek, Milton NY) into liquid nitrogen, followed by lyophilization as previously described[26]. For example, the dried Zn-HI powder (100 mg) was added to 5.8 ml of a 0.17 mg/ml solution of the D,L-PLGA (above) in ethylacetate solvent and homogenized for 2 minutes at 8000 rpm with a shear

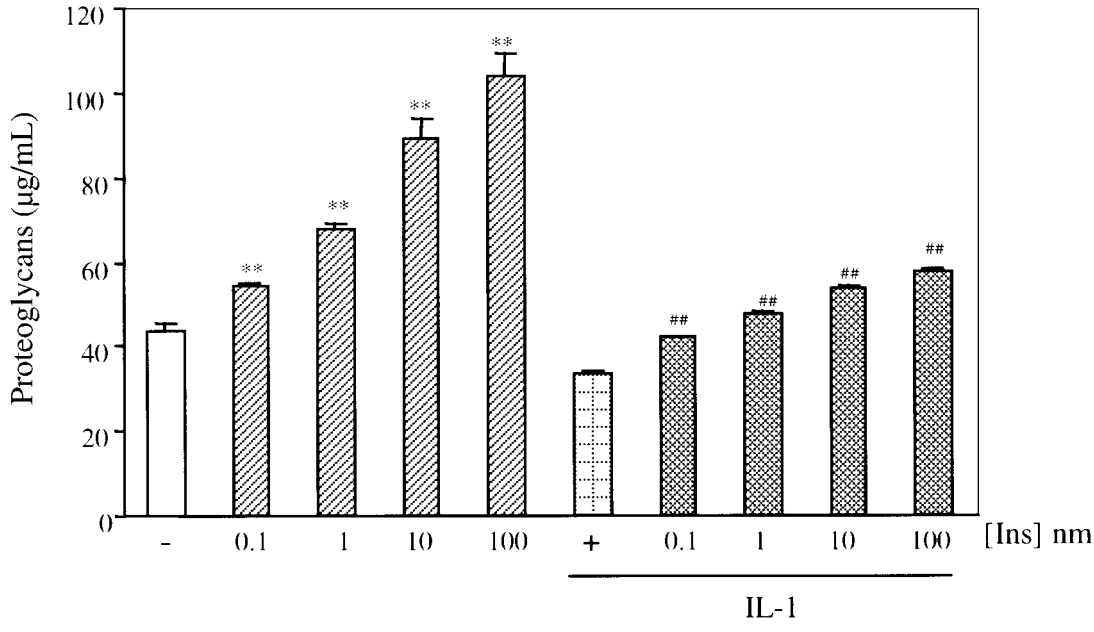

Fig. 1. Proteoglycan synthesis in primary articular chondrocytes. Porcine articular chondrocytes were treated with various concentrations of insulin (0.1, 1, 10, 100 nM), either alone or with IL-1α (1 ng/ml) in serum-free media. Media was analysed for proteoglycan content using a DMMB assay. Data represents the average of four independent samples ± S.E.M. **=P<0.01 for insulin treated vs control. ##=P<0.01 vs IL-1α alone.

homogenizer (Vitrius Inc. Giandiner, NY) to form a uniform suspension of Zn-HI and polymer. The polymer and Zn-HI suspension was sprayed through a sonicating nozzle (Sono-Tek, Milton NY) into a vessel containing 300 ml of frozen ethanol. The vessel was then placed in a −70°C freezer whereupon the frozen ethanol melted and microspheres slowly hardened as the ethylacetate solvent was extracted by the ethanol. After 3 days, hardened microspheres were harvested by filtration (20 μm screen) then dried under nitrogen gas for 4 days and finally sieved (60 μm screen).

PROTEIN ANALYSIS

Encapsulated human insulin was recovered from the microspheres by dissolving in 1.0 N sodium hydroxide (NaOH) and analyzed by UV absorbances.

CHROMATOGRAPHY

To determine the physical integrity of insulin recovered from the compositions, Size exclusion (SEC) and reverse-phase chromatography (RPC) procedures were performed as previously described[27]. Briefly, SEC was performed on a Zorbax® Column with phosphate buffer as the mobile phase. Human insulin was detected by UV absorption at 214 nm. Reverse-phase chromatography was carried out on a C-18 reversed-phase column using sulfate buffer with acetonitrile as the mobile phase at 40°C, human insulin was detected by UV absorption at 214 nm as previously described[27].

RELEASE OF INSULIN IN BUFFER

Twenty mg of each human insulin microsphere formulation was placed in 500 μl of release buffer (10 mM Histidine, 10 mM NaCl, 0.02% Polysorbate 20, 0.02% NaN_3, pH 7.2) and incubated at 37°C to evaluate the *in vitro* release profile for human insulin microsphere formulations. The entire release medium was replaced at each sampling interval with resulting release samples stored at 4°C prior to analysis.

RELEASE OF INSULIN IN SYNOVIAL FLUID

Synovial fluid was harvested from 7–8-week-old male Sprague-Dawley rats and diluted 1:2 with phosphate buffered saline (PBS). The entire release medium was replaced at each sampling interval with resulting samples stored at 4°C prior to analysis.

EFFECT OF PLGA-INSULIN MICROSPHERES ON PROTEOGLYCAN RELEASE AND SYNTHESIS

Explants were treated with PLGA-Ins microsphere resuspended in (Explant Media) on day 0, and media was harvested and replaced (without microspheres) on subsequent days. Other samples in explant media (see above) were treated with fresh insulin (10 nM) at each media change (i.e. at 0, 24, and 48 h timepoints). Proteoglycan breakdown and synthesis were measured as described above.

INTRA-ARTICULAR INJECTIONS

PLGA-Ins microspheres were resuspended in 500 μl of buffer (0.1% hyaluronic acid in PBS), and 3 μl of this solution was injected intraarticularly into mouse joints. As a control, 3 μl of buffer was injected into the contralateral knee. Three days later, proteoglycan synthesis was measured in a patella assay.

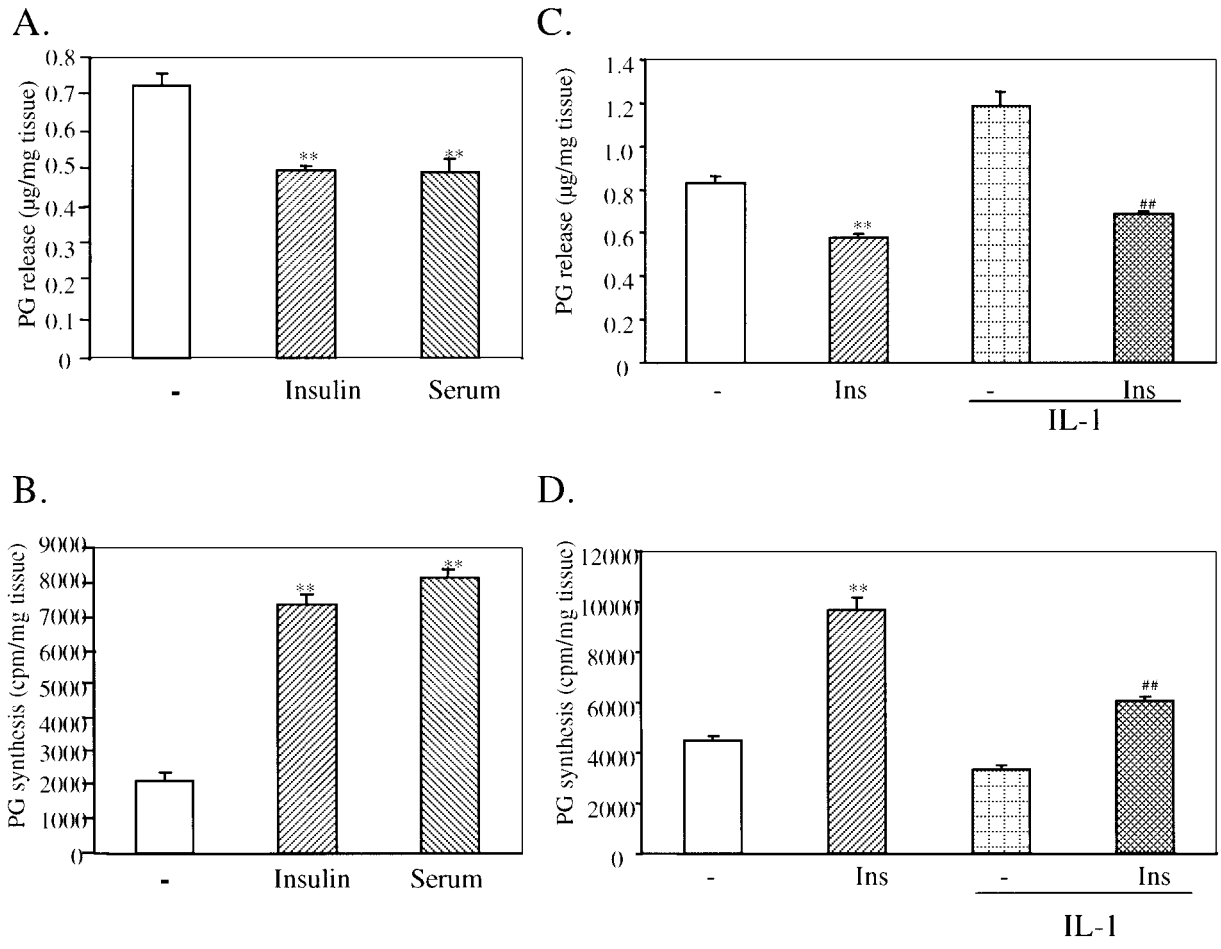

Fig. 2. Matrix turnover in porcine articular cartilage. Porcine articular cartilage explants were treated with Insulin (10 nM) or (a), (b) fetal bovine serum (10%) alone or (c), (d) with IL-1α (1 ng/ml) and the amount of (a), (c) proteoglycan release and (b), (d) proteoglycan synthesis was examined. Data represents the average of five independent samples±S.E.M. **=P<0.01 for insulin or serum treated vs control and ##=P<0.01 vs IL-1α alone.

STATISTICAL ANALYSIS

Differences between experimental groups were tested using a Student's *t*-test. *P* values were adjusted using a Bonferroni correction for multiple comparisons[28]. As indicated in figures, *=*P*-adjusted <0.05, and **=*P*-adjusted <0.01.

Results

INSULIN INCREASES PROTEOGLYCAN SYNTHESIS IN PORCINE PRIMARY CHONDROCYTES

Primary porcine chondrocytes in explant media were treated with insulin at 0.1, 1, 10, 100 nM in the absence or presence of IL-1α at 1 ng/ml. Insulin increased proteoglycan (PG) synthesis in basal and IL-1 treated cultures in a dose-dependent manner (Fig. 1). Enhancement of synthesis was more pronounced under basal conditions. For example, insulin at 100 nM increased basal PG synthesis by 140% but increased that of IL-1 treated cultures by only 70%. Insulin, at a concentration as low as 0.1 nM, was able to overcome the inhibitory effect of IL-1α on PG synthesis (Fig. 1). The ability of insulin to counteract the detrimental effects of IL-1 makes insulin an attractive candidate for the treatment of arthritis in which high levels of IL-1 are implicated in disease progression.

EFFECT OF INSULIN ON ARTICULAR CARTILAGE EXPLANTS

In porcine articular cartilage explants, insulin at 10 nM decreased matrix breakdown by 30% [Fig. 2(a)] and increased matrix synthesis by 240% [Fig. 2(b)] with efficacy equivalent to that of 10% serum [Fig. 2(a),(b)]. In addition, insulin suppressed IL-1 induced catabolism [Fig. 2(c)] and overcame the inhibitory effect of IL-1 on proteoglycan synthesis [Fig. 2(d)] at concentrations as low as 0.33 nM (data not shown).

To better understand the mechanism whereby insulin reduced cartilage matrix breakdown, we analyzed proteoglycan degradation products released from articular cartilage explants. Western blot analysis of aggrecan fragments released into the media from treated tissues was performed using antibodies which recognize either the new NH_2 terminal ARG generated by aggrecanase, or the new NH_2 terminal FFG generated by MMP activity[21]. No MMP-generated fragments were found in explants treated with IL-1, unlike the MMP-generated neo-epitopes

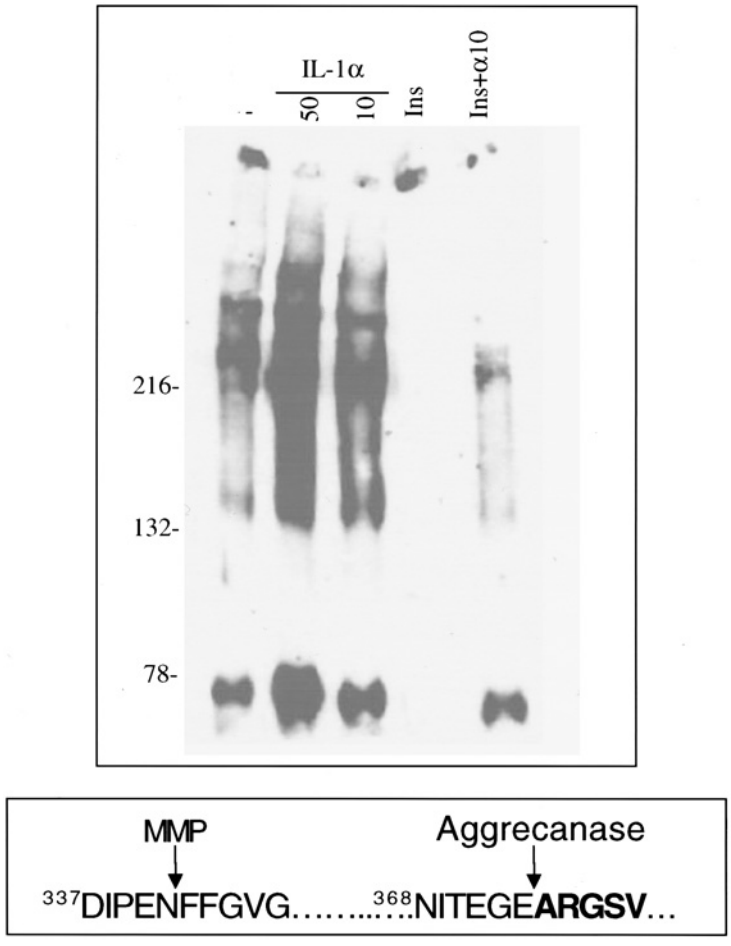

Fig. 3. Western blot analysis of aggrecan fragments generated in articular cartilage explants. Bovine articular cartilage was incubated for 72 h in the presence of insulin (10 nM), IL-1α (50 or 10 ng/ml) or insulin (10 nM) plus IL-1α (10 ng/ml) and aggrecan fragments in the culture media were analyzed by probing a Western blot with an antibody which recognizes the new NH_2 terminal (ARG)[21] produced by aggrecanase cleavage (diagram).

found in explants treated with the MMP activator p-aminophenylmercuric acetate, APMA[29] (data not shown). In contrast, aggrecanase generated fragments were found in basal and IL-1 treated samples[29] (Fig. 3). The high molecular mass band at ~230 kDa could be formed by initial cleavage at the Glu^{373}-Ala^{374}, and additional cleavage in the C terminus at other sites could create the smaller products. Insulin decreased basal and IL-1 induced production of these aggrecanase generated termini (Fig. 3). Thus, insulin inhibited aggrecanase activity, present in articular cartilage explants under serum-free conditions and in the presence of IL-1.

In addition to its ability to decrease matrix breakdown and increase matrix synthesis, insulin also inhibited nitric oxide (NO) production by as much as 40% (at 100 nM) in basal or IL-1α- (1 ng/ml) treated articular cartilage explants (Fig. 4). This effect was seen at concentrations of insulin as low as 1 nM and was also observed in IGF-I treated explants (Fig. 4).

EFFECT OF INSULIN ON GUINEA-PIG ARTICULAR CARTILAGE

Two factors known to affect cartilage matrix metabolism are age and disease. Not only does the rate of biosynthesis and the ability to repair tissue decrease with age, but disease is also associated with a decrease in growth factor sensitivity. More specifically, the response of arthritic cartilage to IGF-I is significantly blunted relative to age-matched controls[5,7,8,30,31].

Because the highly reproducible pattern of cartilage breakdown in Dunkin Hartley (DH) guinea pigs is similar to that seen in the human disorder, the DH guinea pig is a well-accepted animal model for OA[32–36]. For this reason, we tested the effect of insulin on cartilage from guinea pigs at various ages, and thus various stages of degeneration. Insulin increased proteoglycan (PG) synthesis to approximately the same extent (~two-fold) in articular cartilage from 1–2 months (90%) [Fig. 5(a)], 6 months (110%) [Fig. 5(b)], or 11 months (130%) [Fig. 5(c)] old DH guinea-pigs. Thus, insulin is anabolic for articular cartilage from DH guinea pigs at various ages and stages of disease.

Besides increasing proteoglycan synthesis, insulin also decreased matrix breakdown, as shown by a decrease in the amount of proteoglycans in the media of insulin-treated explants [Fig. 5(d)]. Most importantly, the insulin-induced decrease in breakdown and increase in synthesis resulted in a significant net gain in the amount of proteoglycans remaining in insulin-treated cartilage, even in tissue from 11-month-old animals treated for only 3 days [Fig. 5(d)].

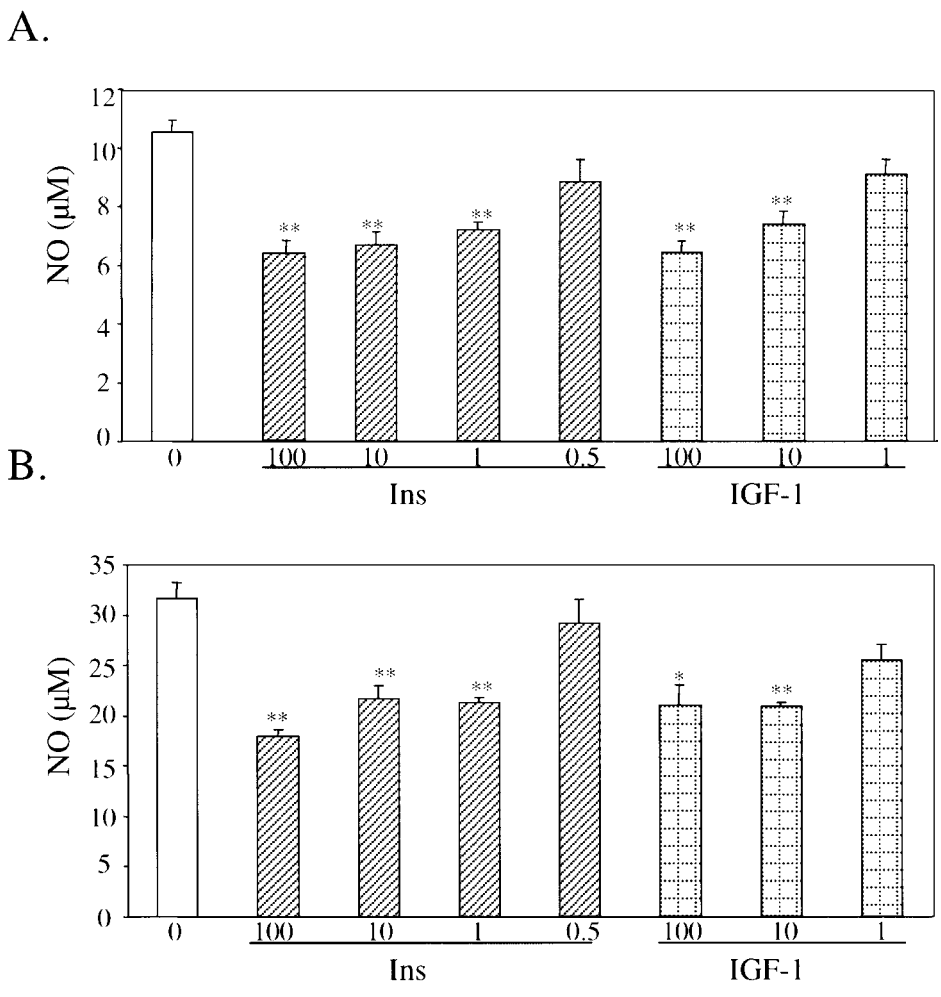

Fig. 4. NO production by articular cartilage explants. Porcine articular cartilage was treated with insulin (100, 10, 1, 0.5 nM) or IGF (100, 10, 1 ng/ml), either (a) alone or (b) with IL-1α (1 ng/ml), and the amount of nitric oxide in the culture media was measured. Data represents the average of five independent samples±S.E.M. *=P<0.05 and **=P<0.01 vs control (0).

EFFECT OF INSULIN ON CARTILAGE FROM DIABETIC MICE

To better understand the effects of diabetes on cartilage matrix metabolism and to test the effects of insulin in another model of diseased, potentially growth-factor resistant cartilage, we measured matrix synthesis in articular cartilage from streptozotocin (STZ)-induced diabetic mice cultured alone or in the presence of insulin. Articular cartilage from severely diabetic, STZ-treated mice had lower basal levels of proteoglycan synthesis than untreated control mice (data not shown) or mildly diabetic, STZ-treated mice (Fig. 6). This decrease in synthesis could be due to the fact that STZ-treatment results in low serum insulin levels due to destruction of pancreatic cells which produce insulin[37]. Insulin treatment increased matrix synthesis (approximately two-fold) in cartilage from mild and severely diabetic mice (Fig. 6). In contrast, IGF-I is not able to increase proteoglycan synthesis in cartilage from STZ-treated animals[38]. Therefore, diseased tissue may not be equally responsive to anabolic factors, and insulin may prove to be more effective than IGF-I in promoting skeletal repair in such individuals.

EFFECT OF INSULIN ON OTHER CELL TYPES

Our data suggest that local delivery of insulin into the joint could be a useful therapeutic for OA. Because several tissues are present within the joint, we tested the effect of insulin on two cell types present in and around cartilage. Bone cells and fibroblasts were treated with insulin or IGF-I at various concentrations, and cell proliferation was measured (Fig. 7). At lower concentrations (0.1 and 1 nM), insulin did not stimulate proliferation of either cell type, unlike IGF-I which stimulated growth of both bone cells and fibroblasts (Fig. 7). Thus, local treatment of a joint with insulin at low concentrations might allow for specific stimulation of cartilage without expansion of bone or synovial fibroblast cell populations.

EFFECT OF INSULIN ON HUMAN ARTICULAR CARTILAGE EXPLANTS

In an effort to compare the activities of IGF-I and insulin on human tissue, we tested their effect on cartilage matrix synthesis in articular cartilage explants harvested from

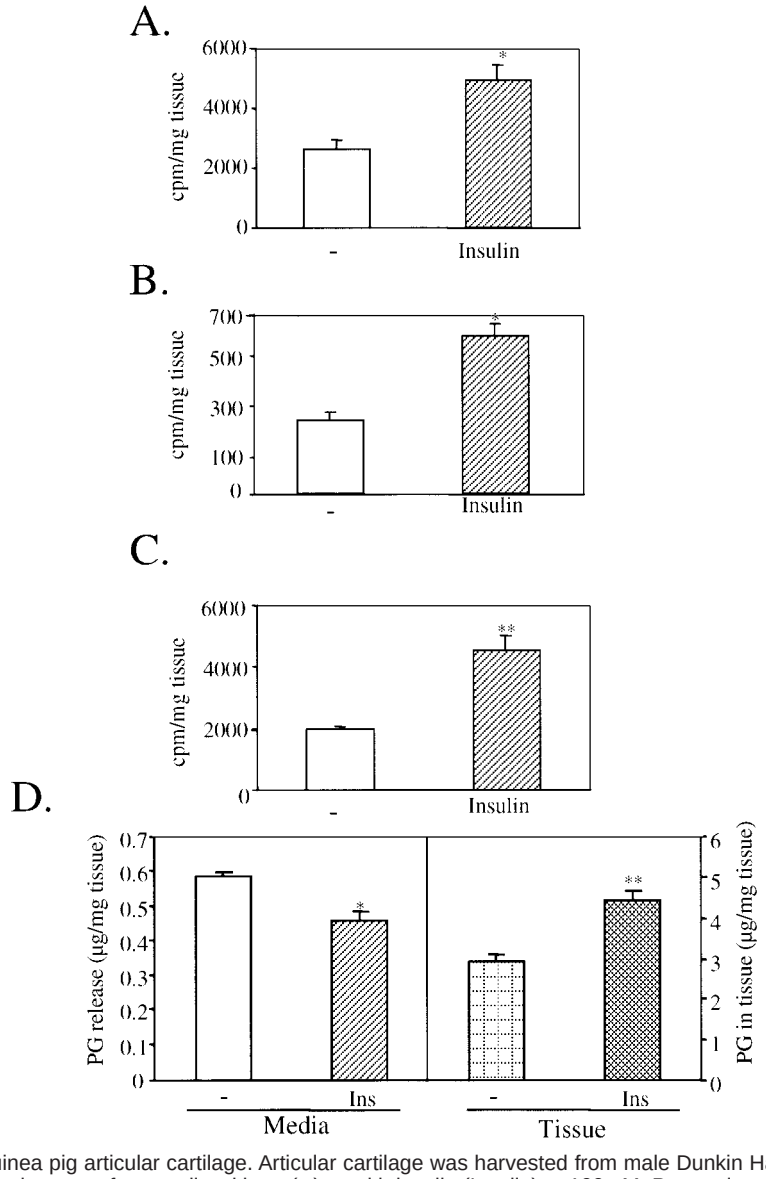

Fig. 5. Effect of insulin on guinea pig articular cartilage. Articular cartilage was harvested from male Dunkin Hartley guinea pigs at different ages and cultured as explants in serum-free media without (−) or with insulin (Insulin) at 100 nM. Proteoglycan synthesis was measured in articular cartilage from guinea pigs which were (a) 1–2 (b) 6 or (c) 11 months of age. (d) Articular cartilage from 11-month-old guinea pigs was treated with insulin (100 nM) (Ins) for 3 days, and proteoglycan release (Media) and proteoglycans remaining in the cartilage (Tissue) were measured. Data represents the average of five independent samples±S.E.M. *=P<0.05 and **=P<0.01 vs control (−).

patients undergoing joint replacement. While matrix synthesis was stimulated by both IGF-I and insulin in tissue from a number of patients, insulin was more active than IGF-I in 20% of the tested articular cartilage samples from osteoarthritic individuals undergoing joint replacement (Fig. 8). Des-(1-3)-IGF-I (desIGF), a naturally occurring variant of IGF-I missing the 3 amino-terminal amino acids, lacks affinity for IGF-BPs, but retains ability to activate IGF-I receptors[39]. Like Insulin, desIGF induced cartilage matrix synthesis in these IGF-I resistant tissues (Fig. 8).

CREATION OF A SLOW-RELEASE INSULIN

In order to establish criteria for a therapeutic formulation of insulin, we tested the stability of recombinant insulin protein. While insulin remained fairly stable when cultured in media at 37°C, addition of articular cartilage led to a dramatic decrease in the amount of active insulin present (Fig. 9). In fact, within 24 h after incubation with cartilage, the amount of active insulin had decreased by ~20%, and by 4 days of culture, the amount of active insulin had decreased by 70% (Fig. 9).

As a first step to increase insulin's stability, the effect of several carriers on articular cartilage was tested. When cartilage explants were divitalized by a brief incubation in base, proteoglycan release and incorporation of ^{35}S-sulfate was completely prevented (data not shown). Thus, our assays are measuring cell-mediated processes. Of the carriers tested, two substantially reduced cartilage matrix synthesis and were therefore excluded as possible carriers (data not shown). We therefore created a slow-release formulation comprised of microencapsulation of a spray-freeze-dried insulin into a matrix of poly-lactic-coglycolic

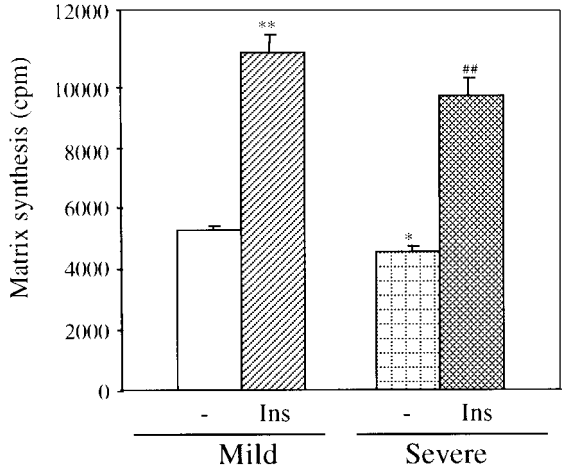

Fig. 6. Effect of insulin on cartilage from diabetic mice. Patellae were harvested from STZ treated mice which had mild (mean glucose= 213 mg/dcl) (Mild) or severe (mean glucose=576 mg/dcl) (Severe) diabetes, and matrix synthesis was measured after culturing in media alone (−) or with 100 nM insulin (Ins). *=P<0.05 and **=P<0.01 vs untreated (−) patellae from mildly diabetic mice, and ##=P<0.01 vs untreated (−) patellae from severely diabetic mice.

acid (PLGA), a material which has been used in other skeletal applications.

SIZE, PROTEIN LOAD AND PHYSICAL INTEGRITY OF THE SLOW-RELEASE FORMULATION

The amount of insulin in the slow-release composition was determined by chemical analysis, and the physical integrity of insulin recovered from the compositions was determined by size-exclusion (SEC) and reverse phase chromatography (RPC). We created two formulations with different insulin to zinc ratios, as defined as moles insulin hexamer/moles of zinc. Formulation I had a ratio of 1:2, while formulation II had a ratio of 1:4. The mean particle diameter distribution of the microspheres was measured on a Malvern Masterisizer X and was found to be 30.2±12 nm for Formulation I and 36.6±11 nM for Formulation II. Protein loading of Formulation I and Formulation II was found to be 5.56% and 5.59%, respectively. Analysis of insulin integrity indicated no significant differences between the protein before and after encapsulation as determined by an insulin kinase receptor activation assay (KIRA) (data not shown).

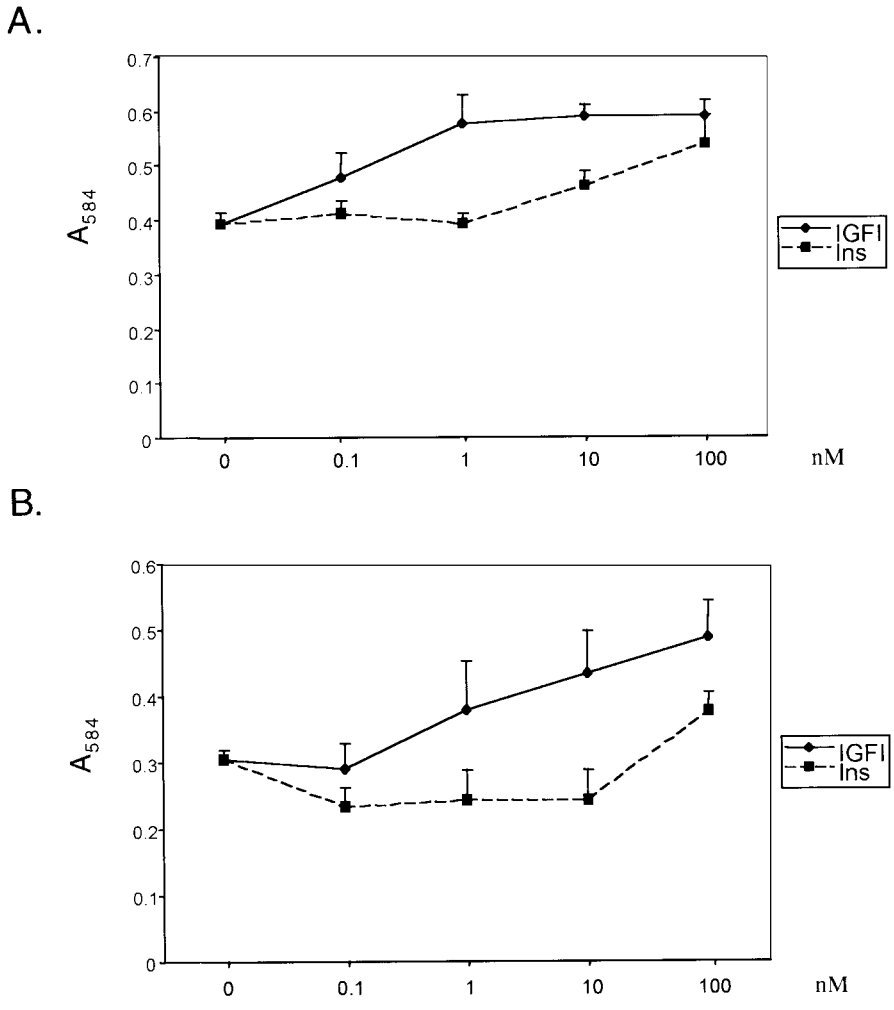

Fig. 7. Effect of Insulin or IGF-I on osteoblast and fibroblast proliferation. (a) MG63 cells (osteoblasts) and (b) NIH3T3 cells (fibroblasts) were treated with Insulin or IGF-I at various concentrations (0.1, 1, 10, 100 nM), and cell proliferation was measured using an MTT assay. Data represents the average of 6 independent samples± S.E.M.

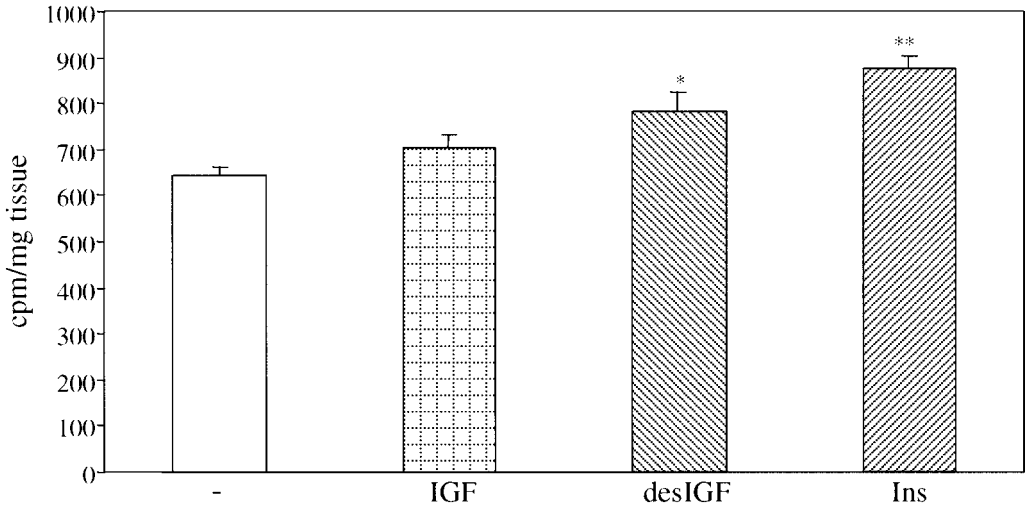

Fig. 8. Response of human OA tissue to IGF-I or Insulin. Human OA tissue (from a 70-year-old female with severe osteoarthritis) was treated with IGF-I (80 ng/ml), desIGF38 (80 ng/ml) and insulin (equimolar concentration), and proteoglycan synthesis was measured. Data represents the average of nine independent samples±S.E.M. *=P<0.05, **=P<0.01 vs control.

RELEASE PROFILE OF INSULIN FROM THE SLOW-RELEASE MATRIX

In order to determine the amount of insulin released, insulin-loaded microspheres were incubated in a histidine buffer or synovial fluid. Samples taken at several timepoints were assayed using an insulin KIRA because of its high sensitivity. An initial burst of release (at day 1) of protein was seen in both buffer and synovial fluid (Fig. 10). The release profiles of insulin indicated a biphasic release pattern with almost 40% of the total loaded protein released in the first 24 h, followed by a lag phase (<1% daily release) and a second release phase (2–5% daily release) over the next 15 days at which point 89% of the total protein loaded had been released [Fig. 10(a)]. Addition of zinc to the formulation appeared to decrease both the magnitude of the initial burst as well as slow the subsequent release [Fig. 10(a)]. After incubation in synovial fluid for 3 days, microspheres from formulation II continued to release active insulin with a concentration of >5 μM [Fig. 10(b)]. Thus, active insulin was released from loaded microspheres, and the formulation process did not seem to be detrimental to protein quality.

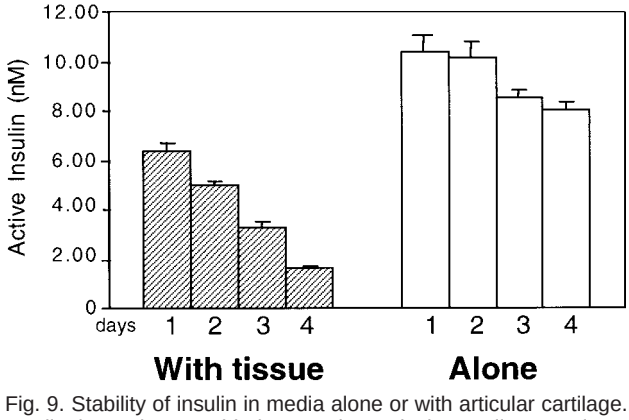

Fig. 9. Stability of insulin in media alone or with articular cartilage. Insulin (10 nM) was added to porcine articular cartilage explants (With tissue) or incubated in serum-free explant media alone (Alone) at 37°C and 5% CO_2. For 4 days, media was tested for insulin activity by an insulin kinase receptor activation (KIRA) assay.

BIOLOGICAL ACTIVITY OF INSULIN AFTER RELEASE FROM THE SLOW-RELEASE MATRIX

To determine the effect a slow-release system might have *in vivo*, insulin-loaded microspheres were tested on articular cartilage explants. PLGA-Insulin (PLGA-Ins) microspheres were added to articular cartilage explants on day 0, and media was harvested and changed (without adding more PLGA-Ins microspheres) every day for 3 days. As a control, other samples were treated with fresh insulin (10 nM) at each media change (i.e. at 0, 24, and 48 h timepoints). Activity was determined by measuring proteoglycan breakdown and synthesis and nitric oxide production. Treatment with PLGA-Ins microspheres decreased matrix breakdown by 30% [Fig. 11(a)] and increased matrix synthesis by 130% [Fig. 11(b)]. In addition, the ability of IL-1α to induce matrix breakdown [Fig. 11(a)] and inhibit matrix synthesis [Fig. 11(b)] was prevented by co-treatment with PLGA-Ins. Finally, PLGA-Ins inhibited both basal and IL-1α induced nitric oxide production by 20% and 30%, respectively [Fig. 11(c)].

IN VIVO EFFECT OF SLOW-RELEASE INSULIN FORMULATION ON MATRIX SYNTHESIS

Data from explant cultures can be useful predictors of *in vivo* effects on articular cartilage. However, within the joint several types of tissue are present, and proteins can be rapidly cleared from the synovial fluid. Therefore, to determine whether or not insulin released from PLGA-Ins microspheres had an effect on cartilage *in vivo*, we injected mouse knee joints with PLGA-Ins microspheres and measured proteoglycan synthesis *in vitro* [Fig. 12(a)].

Three days after injection of PLGA-Ins into mouse knee joints, matrix synthesis was significantly (P<0.05) stimulated relative to the contralateral, buffer-injected joint [Fig. 12(b)]. Even under conditions in which insulin could be

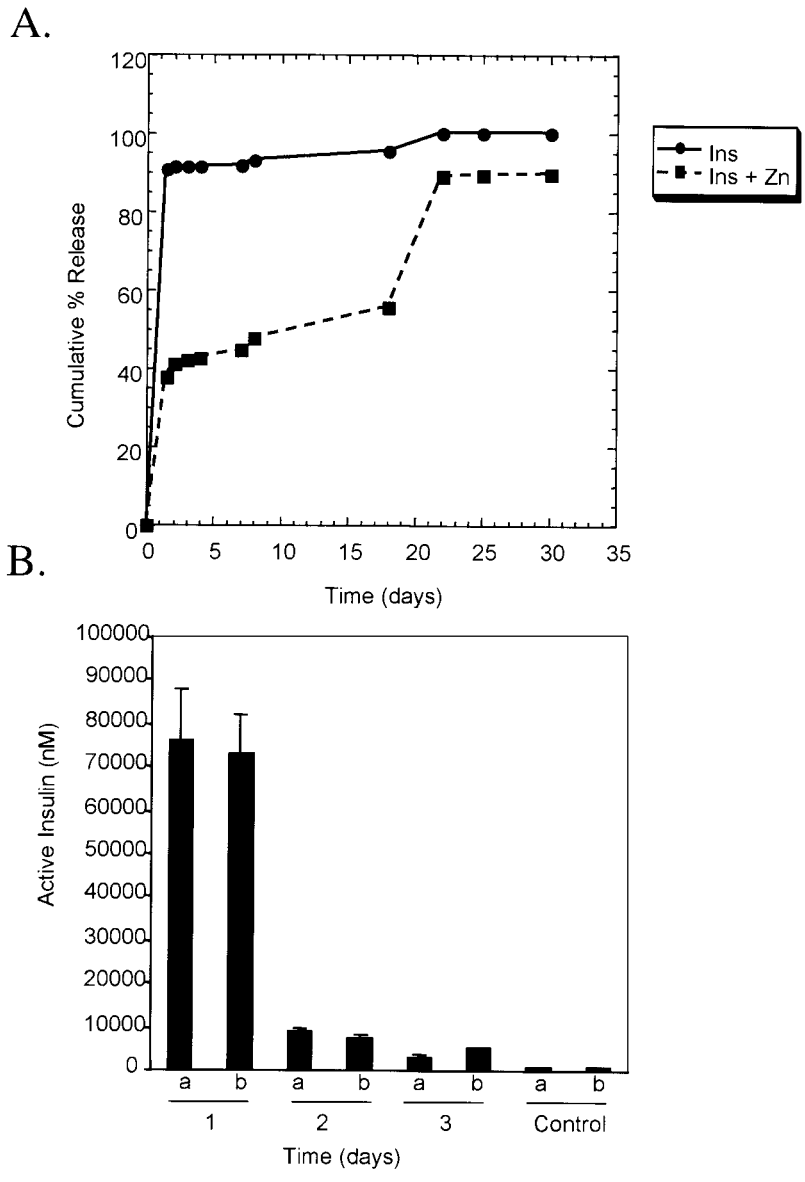

Fig. 10. Release of human insulin from PLGA microspheres. (A) PLGA encapsulated microspheres were placed in 10 mM histidine, 10 mM NaCl, 0.02% polysorbate 20, 0.02% NaN_3, pH 7.2 and incubated at 37°C. Release medium was replaced at each sampling interval, and samples were analyzed for bioactive insulin in an insulin kinase receptor activation (KIRA) assay. (B) Release of insulin in synovial fluid. PLGA-insulin was incubated in synovial fluid harvested from rat knee joints, and duplicate samples were taken daily—day 1 (1a, 1b), day 2 (2a, 2b), and day 3 (3a, 3b)—and tested by a KIRA assay. As a control, synovial fluid spiked with insulin at 500 nM (Control) was also tested. Synovial fluid itself had no detectable insulin activity (data not shown).

cleared from the synovial fluid and/or taken up by surrounding tissues and cells, articular cartilage had an anabolic response to the insulin released by the PLGA-Ins microspheres. The increase observed herein may underestimate the beneficial effects of insulin on cartilage matrix *in vivo*, since the ability of insulin to decrease cartilage breakdown (see Fig. 2) would not have been detected by this assay and thus would not be included in the calculated benefit. The decrease in breakdown induced by insulin would further increase the amount of matrix retained in insulin-treated knee joints. Rather surprisingly, no gross adverse effects were seen upon intra-articular injection of very high doses (30 μg) of insulin once/day for 3 days.

Taken together, our results indicate that insulin remains active after release from microspheres and suggest that PLGA-Ins might be useful as a local, slow-release delivery system for the treatment of cartilage defects.

Discussion

We have shown that insulin had positive effects on matrix metabolism in isolated chondrocytes and articular cartilage explants from pigs, cows, and humans. Insulin stimulated proteoglycan synthesis in isolated primary articular chondrocytes, which are produced by removal of the surrounding extracellular matrix, and may thus be similar to cells found in later stages of cartilage disorders where the matrix has been depleted. In articular cartilage

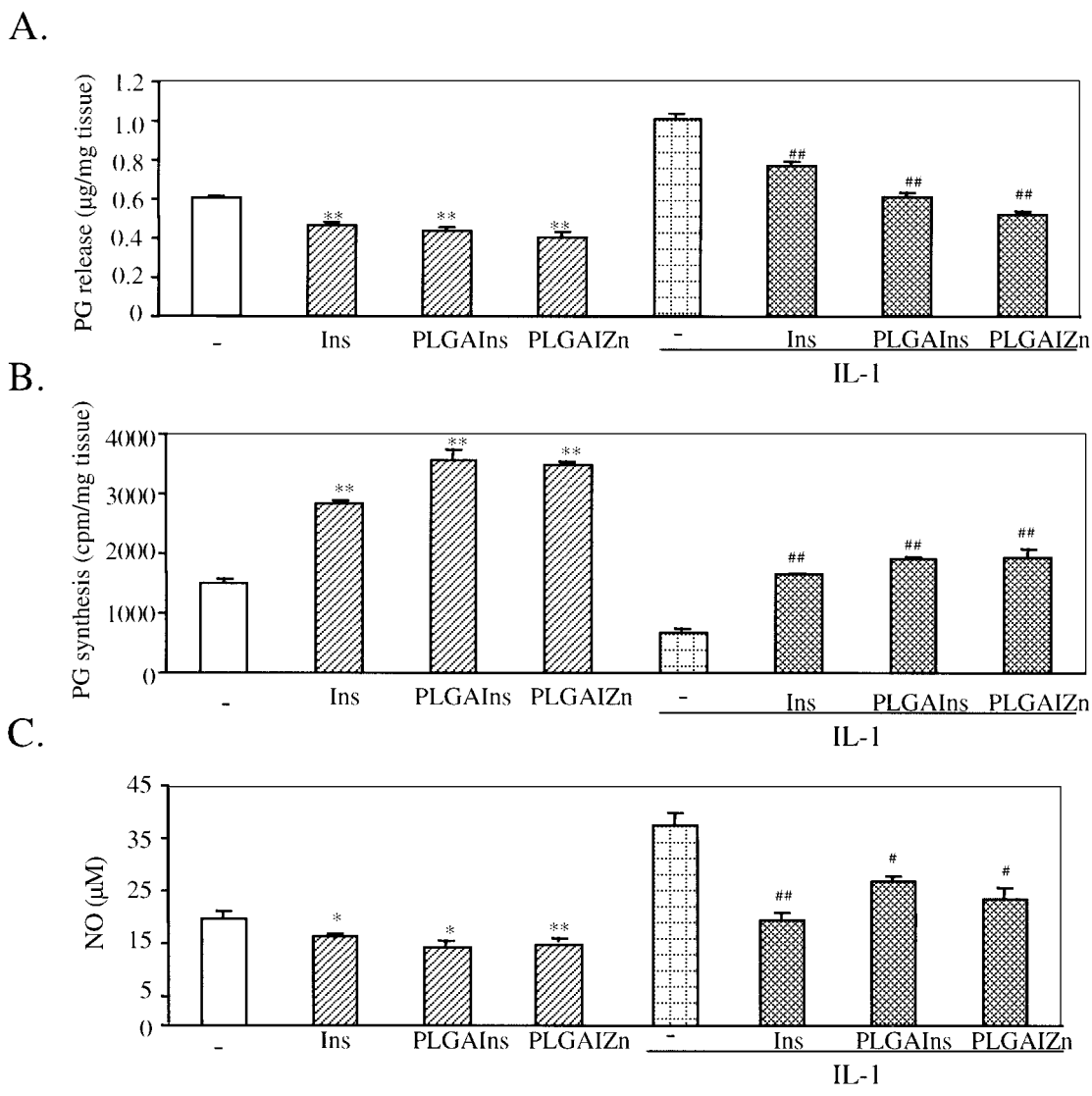

Fig. 11. Effect of slow release insulin on articular cartilage explants. Porcine articular cartilage was treated with PLGA-insulin (PLGAIns) and PLGA-insulin-Zn (PLGAIZn) once (at time 0), in the absence or presence of IL-1α (1 ng/ml) (IL-1). Media was changed at 24 h, and 48 h, without adding additional PLGA-insulin. Samples treated with insulin (10 nM) (Ins) received fresh insulin with the media change at each timepoint (0, 24 h, 48 h). (A) Proteoglycan release (B) proteoglycan synthesis and (C) nitric oxide production were measured. Data represents the average of five independent samples±S.E.M. *=P<0.05 and **=P<0.01 for insulin treated vs control (−). #=P<0.05 and ##=P<0.01 vs IL-1α alone.

explants, in which cells remain embedded in the tissue architecture produced *in vivo*, insulin inhibited matrix breakdown and stimulated matrix synthesis. Our results show that insulin stimulated matrix synthesis in articular cartilage from various ages of Dunkin Hartley guinea pigs which undergo spontaneous cartilage degeneration similar to that of OA[32–36]. Thus, insulin has potential as a treatment for OA at different stages of joint destruction.

Since matrix breakdown is one of the earliest and most destructive features of arthritis, inhibition of this process and stimulation of new matrix molecules would be expected to promote tissue and joint repair *in vivo*[1,2]. High levels of IL-1 are found in arthritic joints, and antagonism of IL-1 function has been shown to reduce the progression of OA[40]. Similarly, nitric oxide can have detrimental effects on chondrocytes as well as other cell types within the joint, and inhibition of nitric oxide can inhibit progression of arthritis in animals[41]. The ability of insulin to counteract the deleterious effects of IL-1 suggests that insulin could inhibit the degradation which occurs in arthritic conditions.

While intra-articular injections are generally well-tolerated by patients and once/week injections of therapeutics are currently being tested, an ideal drug would be one in which a limited number of treatments was required. Unfortunately, human insulin is unstable when stored in neutral solutions at low concentration for extended periods of time[42]. Furthermore, insulin has a half-life of about 5 min in the human body[42,43]. Because insulin stability was dramatically decreased in the presence of articular cartilage, we developed a stabilized, slow-release formulation of insulin. A Zn:human insulin complex is believed to result in a longer-acting formulation of insulin. In fact, histochemical evidence indicates that human insulin is stored in the pancreas as a zinc complex[42,43]. In addition, human insulin complexed with zinc appears to be more resistant to aggregation than uncomplexed human insulin[42]. Finally,

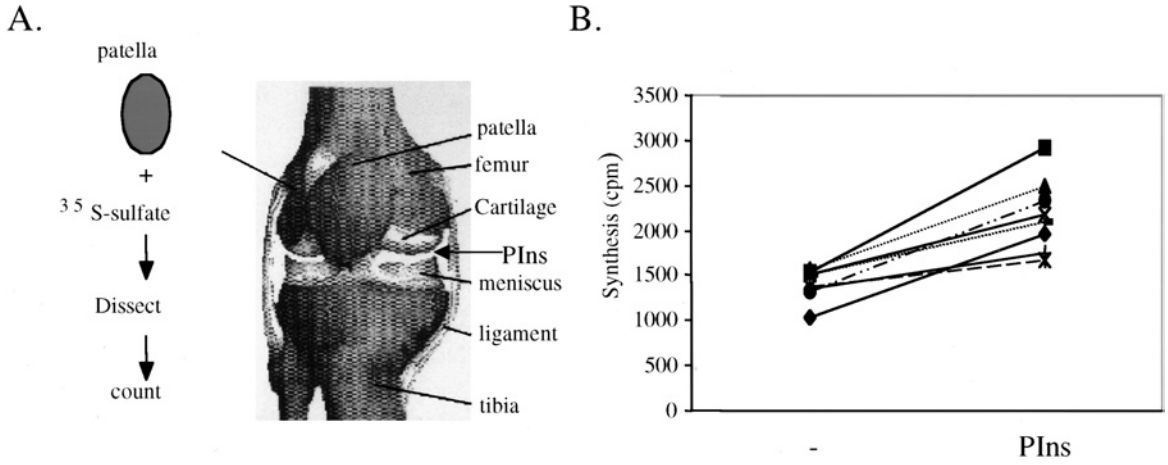

Fig. 12. Effect of slow-release insulin on matrix synthesis *in vivo*. (a) Diagram of the patellae assay. Following intraarticular injection into mouse knee joints, patellae are harvested, labelled with ^{35}S-sulfate, fixed, decalcified, dissected away from the underlying bone, and counted. (b) PLGA-insulin (PIns) was injected into the articular space of the femorotibial joint of the hind leg of mice. Buffer (PBS+0.1% BSA) was injected into the contralateral joint as a control (−). Each line represents a single mouse.

insulin complexed with zinc can have a slower onset and a longer duration of activity (up to 24 h) in humans relative to regular insulin[42]. We created a slow-release poly-lactic-coglycolic acid (PLGA) insulin formulation which was found to be efficacious *in vitro* and *in vivo*. Addition of more zinc to our slow-release poly-lactic-coglycolic acid (PLGA) matrix altered release kinetics.

Patients with diabetes mellitus (DM) have an increased number of musculoskeletal injuries and disorders relative to patients without diabetes[44,45]. In fact, diabetes is one of the known risk factors for developing arthritis. Changes in proteoglycans have been found in the intervertebral disc of diabetic patients, and articular cartilage samples from patients with DM have compromised structural integrity[44,45]. The mechanism underlying these changes in articular cartilage in diabetic patients is not yet known. In animal models of diabetes, systemic changes in insulin (in diabetic or hypophysectomized animals) can result in changes in connective tissues such as skin[13,46] and in aortae[47,48]. Systemic administration of insulin can at least partially reverse the defects in connective tissue metabolism[47–49]. However, these effects of insulin may be due to circulating factor(s) induced by insulin and not due to direct effects of insulin on connective tissues[49]. We found that the defect in cartilage matrix synthesis in diabetic mice could be reversed by transient, local treatment with insulin. These results suggest that insulin deficiencies can lead to abnormally low rates of cartilage matrix synthesis. Furthermore, insulin could be an effective treatment for disorders (other than primary arthritis) in which cartilage tissue has defects in matrix synthesis and/or breakdown.

The metabolic imbalance in osteoarthritis which favors matrix breakdown over new matrix synthesis may be due, at least in part, to insensitivity of chondrocytes to IGF-I stimulation[3–8]. While the mechanism underlying this IGF-I resistance is not known, the elevation of IGF-binding proteins (IGFBPs) in many OA patients may play a role[3–8,30]. Consistent with this hypothesis, we found that articular cartilage from some (20% of the samples tested) of OA patients undergoing joint replacements responded better to insulin and IGF variants[39], which do not bind IGF-BPs, than to IGF-I alone. In these patients, treatment with insulin may prove to be more effective than treatment with other growth factors such as IGF-I.

The mechanism by which insulin decreases matrix breakdown appears to be through inhibition of aggrecanase activity. Furthermore, given that the doses of insulin at which we see efficacy (0.1, 1, 10 nM) are below the concentration necessary to activate IGF-I receptors[17], signaling by insulin in cartilage may occur through activation of insulin receptors. Inhibition of IL-1 activity by insulin may be through production of IL-1 receptor antagonist (IL1ra), since many growth factors, including IGF-I, stimulate this inhibitor[40].

In addition to the cartilagenous changes in OA, subchondral bone thickens and bony nodules (osteophytes) are formed. Given these changes in bony architecture and volume, treatment with a factor which stimulated further bone growth would not be optimal. Unlike IGF-I, which stimulated growth of osteoblasts and fibroblasts, insulin (at low concentrations) was a poor mitogen for these cells. Thus, local treatment of insulin within the joint may be able to selectively target articular cartilage, without altering the surrounding tissues such as bone and synovium.

Taken together, our results demonstrate that insulin mitigated loss of matrix molecules and stimulated synthesis of new molecules to replace those lost. The net effect was to increase the total amount of proteoglycans. These effects of insulin were shown *in vitro* and *in vivo*. By increasing the amount of matrix retained in cartilage, insulin treatment *in vivo* could lead to maintenance of articular cartilage matrix, and thus inhibition of subsequent joint destruction and deformity. As such, insulin when delivered in a sustained release manner is very attractive as a possible therapeutic for arthritic patients.

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